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ABSTRACT

NKLAM (Natural Killer Lytic Associated Molecule) is a novel protein within the cytolytic granules of natural killer (NK) cells and cytotoxic T cells (CTL). Reduction in NKLAM expression decreases the cytotoxicity of these cells, indicating a role of NKLAM in killing. NKLAM possesses three zinc binding RING finger domains, motifs involved in protein-protein interactions. NKLAM has been found to bind to three proteins, URKL-1 (Uridine Kinase Like-1), and E2 ligases UbcH7 (human ubiquitin conjugate 7) and UbcH8. The interactions between NKLAM and URKL-1, as well as the ubiquitin conjugates, have been shown to involve the second cysteine rich domain of NKLAM. The interaction between NKLAM and UbcH8 has also been found to occur endogenously. Confocal microscopy shows that NKLAM and URKL-1 are co-localized in the cytoplasm of 293 cells when they are transfected together, however, transfected separately URKL-1 is found in the nucleus and NKLAM remains in the cytoplasm. We also identify increased degradation of URKL-1 in the presence of NKLAM compared to the level of degradation occurring with the NKLAM constructs. These results solidify our finding that NKLAM acts as an E3 ligase toward URKL-1.

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Introduction

The grant entitled "The Role of NKLAM in the Killing of Tumor Cells" supports research to further characterize the protein NKLAM (Natural Killer Lytic Associated Molecule). NKLAM had been previously identified as a RING finger protein that is necessary for cytolytic activity of natural killer (NK) cells (1,2). We have previously shown that NKLAM interacts with proteins Uridine Kinase Like-1(URKL-1) and human ubiquitin conjugates 7 (UbcH7) and 8 (UbcH8). We have shown URKL-1 interaction occurs primarily with the first and second RING of NKLAM. We have also shown that this interaction resulted in increased ubiquitination of URKL-1. Our co-localization studies using an overexpression system in 293 cells had identified that both proteins are found in the cytosol of the 293 cells. In this report we present data showing that URKL-1 is sequestered in the cytoplasm in the presence of NKLAM, but is found in the nucleus in NKLAM's absence. We also show that the RINGs of NKLAM are involved in ubiquitin conjugate interaction, with the second RING possessing the strongest interaction. We present here that endogenous UbcH8 binds NKLAM in NK cells and show increased degradation of URKL-1 in the presence of NKLAM. We discuss the advancements we have made over the past year in hopes to expanding our knowledge of how NKLAM is involved in the cytolytic activity of NK cells

Body

Task 1 focused on the identification of proteins that interact with NKLAM. As reported with the last update, we confirmed through confocal microscopy that NKLAM and URKL-1 could be found in the same region of 293 cells that were overexpressing both proteins. A previous study involving URKL-1 had found that the protein was localized to the nucleus of their cells (1). Because of this data we wanted to reaffirm the location of URKL-1 and NKLAM when they were overexpressed by themselves in 293 cells. This study was performed by taking 293 cells and co-transfecting them with either a plasmid encoding Flag-URKL-1 or one encoding NKLAM. The transfected cells were fixed onto cover slips and treated with a primary antibody to Flag or to NKLAM followed by the secondary antibodies Alexa 568 and Alexa 488 to identify the presence of URKL-1 and NKLAM respectively. Following the secondary antibody the cells were treated with TOPRO3, a nuclear stain. The results showed that URKL-1, seen in red, was found predominately in the nucleus (Fig 1a), the purple stain shows blue nuclear stain overlapping with the URKL-1 red stain (Fig 1b). The cells that stained only blue are not expressing URKL-1. NKLAM, however, seen in green, (Fig 2a) remained localized to the cytosolic region toward the membrane of the cell when transfected alone (Fig 2b). Again the blue indicated the nuclear region of the cells (Fig 2b). We repeated this experiment and counted the number of cells where URKL-1 was found in the nucleus as well as the number of cells where URKL-1 was found in the cytosol. When counting the cells that were transfected with both NKLAM and URKL-1, we only counted those that expressed both proteins. The results showed that when URKL-1 was transfected alone into 293 cells it was found in the nucleus in 92% of the cells where transfection had occurred. When transfected with NKLAM, however, URKL-1 was only found in the nucleus in 35% of the cells that were co-transfected; in 65% of the co-transfected cells URKL-1 was found in the cytoplasm. NKLAM was found 100% of the time in the cytoplasm regardless of individual or co-transfection. These results show a possible sequestering of URKL-1 in the cytoplasm in the presence of NKLAM.

The studies to complete **task 2,** identifying NKLAM's RING, or cysteine rich domains (CRD)s responsible for protein interaction, were performed using truncated forms of NKLAM ligated into the CS2-MT vector, myc-tagging the NKLAM truncations (Fig 3). This was done to determine which RINGs are responsible for NKLAM's interaction with URKL-1 and the ubiquitin conjugates. As we reported previously, co-transfection of the flag-tagged URKL-1 plasmid along with an NKLAM construct identified the second CRD as possessing the strongest interaction with NKLAM. We also showed that the first CRD was able to interact with URKL-1 as well. The third CRD of NKLAM, however, possessed a very weak interaction with URKL-1. We continued our studies of the RING domains of NKLAM using the NKLAM constructs and co-transfecting them with a Flag-UbcH7 or Flag-UbcH8 plasmid into 293 cells. We performed immunoprecipitation assays using a myc antibody, pulling down the protein made by the NKLAM construct. Western analysis followed using the Flag antibody to identify the presence of the ubiquitin conjugate. These co-precipitation experiments have confirmed our preliminary findings. The interaction between UbcH7 and NKLAM was strongest with the second CRD (Fig 4a). The first CRD was able to interact with UbcH7, but less UbcH7 was precipitated out in its presence. UbcH7, however, was unable to be precipitated in the presence of the third CRD alone (Fig 4a). Co-precipitation studies performed using UbcH8 and the truncations of NKLAM show a similar binding pattern to that of UbcH7. Our co-precipitations of UbcH8 and the NKLAM constructs showed less interaction between NKLAM's third CRD compared to the first or the second CRD (Fig 4b). Similar to the UbcH7 interaction, the second domain appeared to have the greatest interaction with UbcH8 (Fig 4b). These binding results suggest that the second CRD is the most important in NKLAM's interaction with URKL-1 but also with the ubiquitin conjugates. Although the third CRD may not be directly involved in the interaction with URKL-1 or the ubiquitin conjugates, we believe it supports NKLAM's unique three dimensional structure allowing for specific proteins to interact.

Since we were able to show that protein interactions occurred between NKLAM and the ubiquitin conjugates in the overexpression system we attempted to identify the same interaction occurring endogenously in NK cells. To do this experiment we stimulated NK3.3 cells with IL-2 and IFNβ to upregulate NKLAM and UbcH8 expression (2,3). MG132 was then added to decrease protein degradation of NKLAM. We then extracted proteins from the cells using lysis buffer containing .5%NP-40 along with protease inhibitors and iodoacetamide. The lysates were subjected to immunoprecipitation using a NKLAM specific antibody followed by Western analysis using an antibody against UbcH8 to identify its co-precipitation with NKLAM (Fig 5). Lysates were also subjected to Western analysis to ensure both proteins were present. The results showed that UbcH8 co-precipitated with NKLAM. This then confirmed the protein interaction we observed in the overexpression system, reinforcing NKLAM as an E3 ligase.

Since NKLAM's interaction with the ubiquitin conjugates suggests it functions as an E3 ligase, a protein involved in the ubiquitination of a substrate (4,5), we performed tests to determine if URKL-1 degradation was enhanced in the presence of NKLAM. We co-transfected 293 cells with URKL-1 along with either myc-NKLAM or the NKLAM constructs CRD1,2,3, CRD1, CRD2, CRD3. After the transfection cell lysates were obtained and Western analysis was performed using the Flag antibody to identify the presence of URKL-1. The results showed that when the entire protein of NKLAM was expressed alongside URKL-1, the rate of URKL-1 degradation was much greater compared to the rate of URKL-1 degradation seen when it was co-

transfected with a construct NKLAM (Fig 6). These results strongly suggest that NKLAM functions as an E3 ligase and URKL-1 acts as its substrate.

In order to further characterize NKLAM we tested whether it is self ubiquitinated like other known E3 ligases (6.7). We had previously stated that our attempts to show NKLAM is self ubiquitinated were inconclusive. Since that time we have been able to show ubiquitination of NKLAM in 293 cells that were co-transfected with myc-NKLAM and HA-His. We obtained the cell lysates from these cells and pulled down NKLAM followed by Western analysis using an HA antibody to identify the presence of ubiquitin. We were able to show that NKLAM is ubiquitinated, but we also observed that ubiquitination does not occur with the form of NKLAM that possesses only the third cysteine rich domain (Fig 7). We believe the inability of the CRD3 construct to be ubiquitinated is due to the fact that it does not bind well to the ubiquitin conjugates. Since the ubiquitin chains are attached to the ubiquitin conjugates, their inability to bind to the NKLAM construct would prohibit these ubiquitin chains from coming into close proximity to the CRD3 protein.

Task 3 was to determine if overexpression of the mutated NKLAM affects the cytotoxic function of NK cells. We have attempted additional experiments using the Nucleofector to transfect NK cells. Although our numbers of transfections remain small, the experiments are ongoing. In addition to the transfection experiments we have also attempted to find endogenous NKLAM in NK3.3 cells using confocal microscopy. We initially faced obstacles of high background; we believe this is a result of the Fc receptors expressed on the NK cell membrane. We have performed studies using goat serum as a blocking agent and have had some success. These studies are presently ongoing as we attempt to find NKLAM in NK3.3 cells despite its low expression level.

Key Research Accomplishments

- Determined URKL-1 is localized in the nucleus without the presence of NKLAM, and localized to the cytosol in NKLAM's is presence.
- Determined CRD2 provides the greatest interaction between NKLAM and the ubiquitin conjugates.
- Identified NKLAM and endogenous UbcH8 interaction in NK3.3 cells.
- Determined degradation of URKL-1 is enhanced in the presence of NKLAM
- Determined NKLAM is ubiquitinated when overexpressed in 293 cells, and that ubiquitination is abrogated when only the third CRD of NKLAM is present.

Reportable outcomes:

A manuscript detailing the protein interaction between NKLAM and the ubiquitin conjugates was submitted Nov 2004 to JBC. This manuscript was rejected; it has since been edited and is being resubmitted. A copy of the initial manuscript was sent to DoD in Nov 2004.
Poster presentation was presented at ERA of hope 2005. Title: Characterization of NKLAM as an E3 ligase.

Conclusions:

Our results show that the RING domains of NKLAM are involved in the protein-protein interaction with ubiquitin conjugates UbcH7 and UbcH8. We were able to show that the second

RING domain possesses the strongest interaction between NKLAM and each of the conjugates, and that the third domain's interaction is the weakest, and possibly non-existent with UbcH7. These data are similar to the binding results we had previously observed with NKLAM and URKL-1. The similarity in binding suggests that the ubiquitin conjugate and the substrate may not bind to the E3 ligase NKLAM simultaneously. This then would suggest that NKLAM would hold unto the ubiquitin chain before transferring onto the substrate. We were also able to confirm that endogenous NKLAM is able to interact with endogenous UbcH8. This study reinforces our findings of the conjugate NKLAM interaction we observed in the overexpression system, but affirms our suggestion that NKLAM is an E3 ligase.

The belief that NKLAM functions as an E3 ligase is confirmed with the increased rate of degradation we observed with URKL-1 in the presence of NKLAM. The same level of degradation was not observed with URKL-1 when it was co-transfected with the constructs of NKLAM that did not express a complete protein. We believe that the decrease in degradation is due to insufficient binding to either URKL-1 or the ubiquitin conjugates.

Thus our studies have shown that NKLAM functions as an E3 ligase and that it is able to bind to ubiquitin conjugates UbcH7 and UbcH8, and these conjugates help to facilitate the ubiquitination of NKLAM's substrate URKL-1. Studies are continuing that will ensure the interaction between NKLAM and URKL-1 occurs endogenously. They are also proceeding to determine where NKLAM and URKL-1 interact in the NK cell. It is hoped that these data presented here, and identified in the future involving NKLAM will help to regulate the cytolytic activity of NK cells, a possible defense against breast cancer or metastases.

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6. G. Sobue et al. Biochem. Biophys. Res. Commun. 281, 706-713 (2001).

7. E. Fon et al. J. Biol. Chem. 277, 486-491 (2002).

Appendix

Figure 1

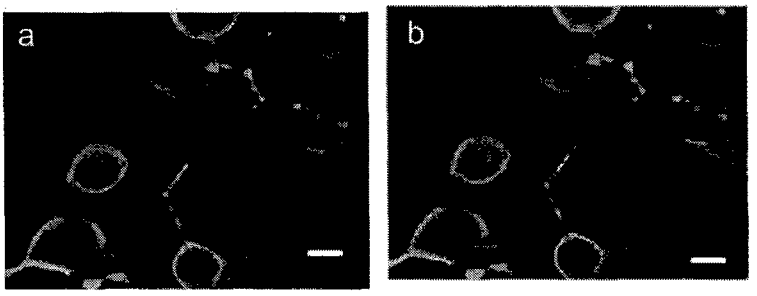


Figure 1: Localization of NKLAM in 293 cells.
293 cells that were transfected with NKLAM were fixed onto microscope cover slips. The cover slips were then incubated with primary antibody 14 to indicate the presence of NKLAM followed by secondary antibody Alexa 488. The cells were then treated for 10min with TOPRO3, a nuclear staining reagent. a and b) NKLAM shown in green is located near the cell membrane b) and is absent from the cell nucleus, seen in blue.

Figure 2

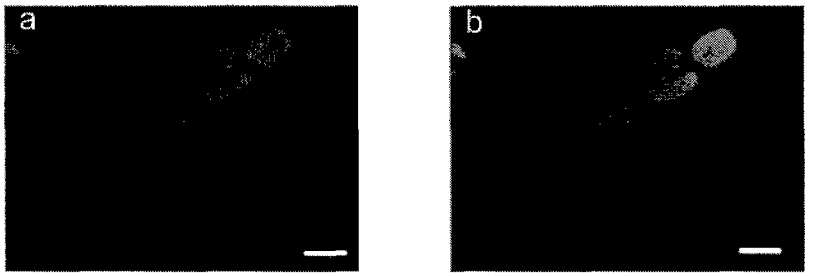


Figure 2: Localization of URKL-1 in 293 cells.
293 cells that were transfected with URKL-1 were fixed onto microscope cover slips. The cover slips were then incubated with primary Flag antibody to indicate the presence of URKL-1followed by secondary antibody Alexa 568. The cells were then treated for 10min with TOPRO3, a nuclear staining reagent. a) URKL-1 seen in red is located in the nuclear region of the cell. b) Purple indicates the location of URKL-1 coincides with the TOPRO3 staining of the nuclei. Scale bar indicates 10uM

Figure 3

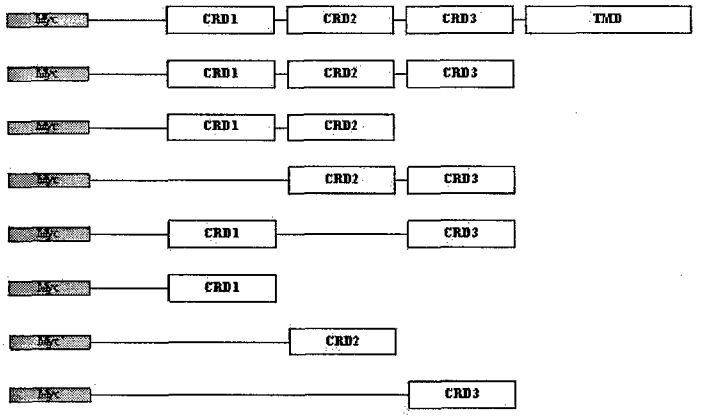


Figure 3: Design of NKLAM constructs. NKLAM constructs containing one or more CRDs were designed using DNA fragments and PCR products obtained from NKLAM cDNA. The 5' region of NKLAM was obtained by restriction digestion of the cDNA. This was ligated to the appropriate PCR products containing the desired CRDs. The ligated fragments were then inserted into the CS2-MT vector, myc tagging the NKLAM constructs. The numbers following the CRD notation indicates the cysteine rich domains present in the constructs. All CRD NKLAM constructs lack the transmembrane domain.

Figure 4a

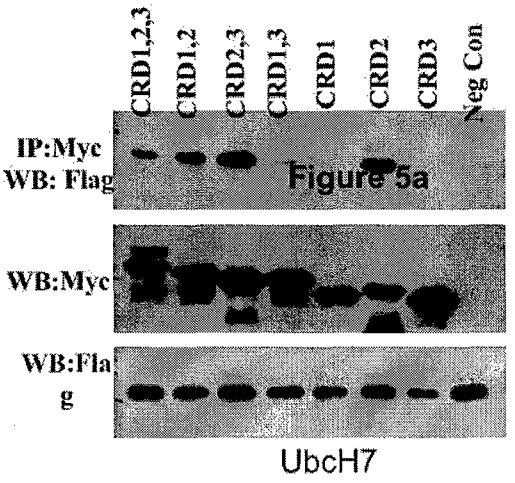


Figure 4b

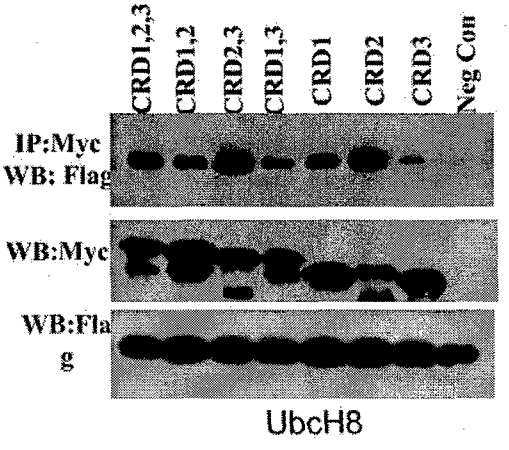


Figure 4: Co-immunoprecipitation of UbcH7 and UbcH8 with NKLAM CRDs.
293 cells were co-transfected with a plasmid encoding a myc tagged NKLAM CRD construct and either Flag-tagged UbcH7 (Fig 4a) or UbcH8 (Fig 4b). 24h post transfection cells were treated with MG132 and 16h later lysates were obtained. The lysates were mixed with 2ug monoclonal myc Ab to precipitate NKLAM. The precipitates were resolved by SDS-PAGE and subjected to Western analysis using polyclonal Flag Ab to identify the ubiquitin conjugates. Samples of the cell lysates were also subjected to Western analysis to ensure the presence of NKLAM and the conjugate proteins in the lysates. Monoclonal myc Ab was used to identify NKLAM; polyclonal Flag Ab was used to identify both UbcH7 and UbcH8.

Figure 5

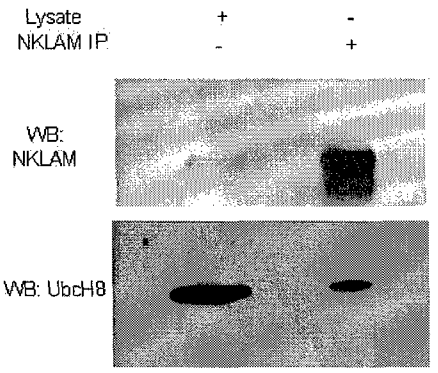


Figure 5: Immunoprecipitatoin of endogenous UbcH8 with NKLAM
NK3.3 cells were stimulatd with IL-2 and IFNβ followed by the addition of MG132. Cell lysates fwere obtained and mixed with NKLAM antibodies 14, 35, and 76, along with protein G. The cell lysates and the resulting precipitates were subjected to Western analysis using a UbcH8 specific antibody to identify the presence of UbcH8. The

Figure 6

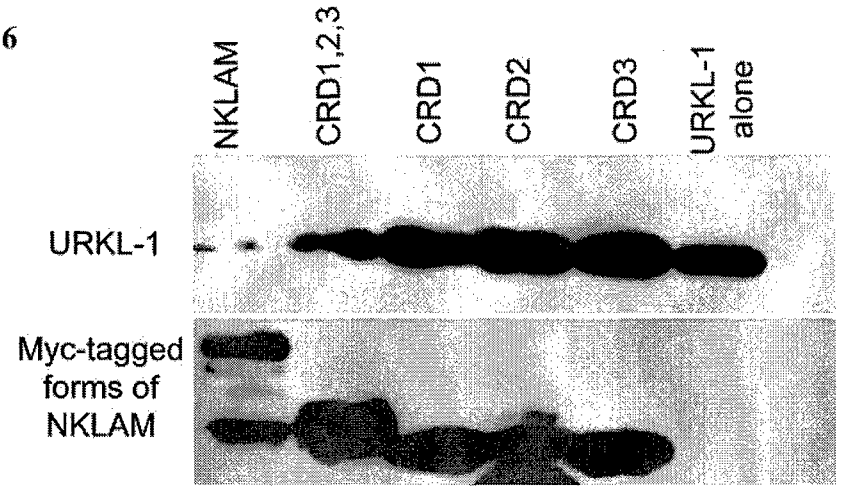
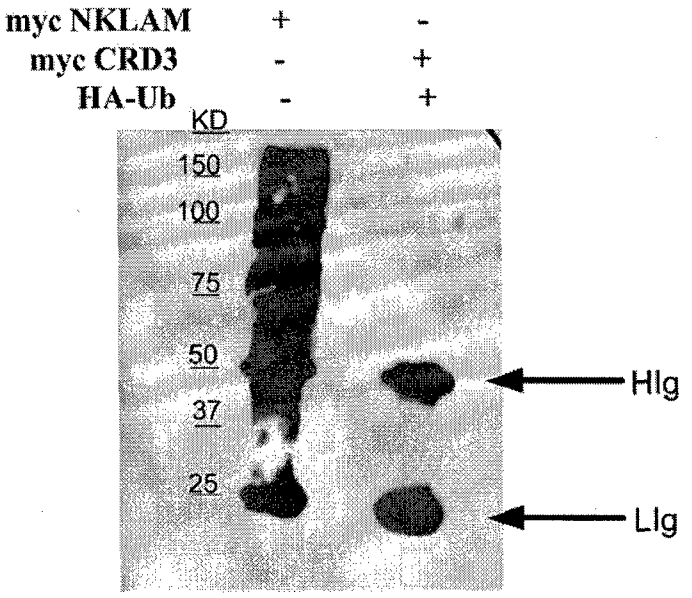


Figure 6: URKL-1 protein expression in the presence of NKLAM
293 cells were co-transfected with Flag-URKL-1(all lanes) along with myc-tagged full length NKLAM, myc-CRD1,2,3, myc-CRD1, myc-CRD2, and myc-CRD3, as well as with Flag-URKL-1 alone. 24h post transfection cell lysates were obtained and the lysates were subjected to Western analysis using polyclonal Flag antibody to identify the expression level of URKL-1. Western analysis was also performed on the lysates using monoclonal myc antibody to identify the presensce of the NKLAM forms.

Figure 7



IP: Mono myc Ab
WB: Anti-HA

Figure 7: Ubiquitination of NKLAM. Plasmids encoding myc-NKLAM, myc-CRD3, and HA-ubiquitin were co-transfected into 293 cells. 24h post transfection, cells were treated with 2uM MG132 for 16h. Lysates were mixed with monoclonal myc antibody and protein G to precipitate NKLAM and its shortened form. After SDS-PAGE immunoblotting was performed using HA antibody to identify the presence of ubiquitin. Only the heavy and light immunoglobulin chains were present.